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COMMUNICATION

High-throughput evaluation of interactions between biomaterials, proteins and cells using patterned superhydrophobic substrates†

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We propose a new low cost platform for high-throughput analysis that permits screening the biological performance of independent combinations of biomaterials, cells and culture media. Patterned superhydrophobic flat substrates with controlled wettable spots are used to produce microarray chips for accelerated multiplexing evaluation.

Surface aspects play an essential role in dictating the biological performance of implantable biomedical devices. Surface characteristics such as surface chemistry, exposed biochemical signals and physical/topological features should be considered in the design of biomaterials that will interact with proteins, cells and tissues.[1] The complete picture of cell–biomaterial relationships is far from being understood, due to the existence of inter-dependencies between the different surfaces properties, the influence of the cell type and the complex effect of protein adsorption.

High-throughput screening (HTS) approaches permit to correlate the characteristics of materials, surfaces and the biological responses, including cell adhesion, growth and differentiation or gene expression in a single experiment.[2] Different libraries/methods have been employed in such HTS, including surfaces varying in roughness, surface chemistry/energy, mechanical properties and density of biochemical elements.[3] The substrates for such kind of HTS have been fabricated by: robotic DNA spotter; microfabrication masking techniques, such as photolithography, soft-lithography, microfluidics, templating, imprint lithography, microelectronics and magnetic forces; and direct microfabrication techniques such as contact printing and non-contact printing, ink-jet printing, electron beam lithography and dip pen nanolithography.[4] The microarray format enables the rapid synthesis of suitable polymers or deposition of different materials and thereafter screening a large library of multiple biomaterials and microenvironments. However, in the methodologies all the spots employed in the chip are usually tested in the same biological environment that means the entire device is immersed in a unique culture medium. Advances in this field should offer the possibility to screen individually and in the same chip different combinations of biomaterials under different conditions, including different cells, culture media or solutions with different proteins or other molecules.

Here we describe a novel method for rapid, microlitre-scale deposition of biomaterials or proteins and characterization of their interactions with cells based on the use of patterned superhydrophobic surfaces. Inspired by the water repellent behaviour of hierachical micro/nanostructured surfaces found in the nature, such as the lotus leaves, synthetic superhydrophobic surfaces have been produced exhibiting water contact angles higher than 150°.[5] The proposed HTS approach uses patterned superhydrophobic substrates with wettable spots to produce flat microarray chips for multiplexing evaluation of material/cell interactions. The hypothesis is that liquid volumes may be confined in regions due to strong contrasts in surface tension, enabling to deposit with high control materials, cells and other substances. To validate the methodology we investigated the cellular behaviour onto patterned polystyrene (PS) superhydrophobic substrates pre-adsorbed combinations of proteins. Human serum albumin (HSA) and human plasma fibronectin (HFN) were chosen in this work on the basis of their importance in a variety of biomedical and biological aspects and in tissue engineering.[6]

The original smooth PS surface (see Fig. S1A in the ESI†) presented a water contact angle (WCA) of 98.9° ± 1.1°. The rough PS surfaces presented a WCA of 156.2° ± 0.3° were prepared by a phase-separation methodology described before.[7] These surfaces exhibit a hierarchical structure at both nano- and microscales: spheres with sizes from *ca.* 50 nm to 200 nm that are agglomerated in larger micrometre structures (see Fig. S1B in the ESI†). The change in wettability was achieved by exposing the surfaces to UV/Ozone (UVO) irradiation. The evolution of the WCA was measured as a function of UVO irradiation time for both smooth and rough PS surfaces (Fig. 1A). Using such treatment one can control the wettability of the rough surfaces in the entire superhydrophobic to superhydrophilic range. X-Ray photoelectron spectroscopy (XPS) measurements permitted to assess the changes in the chemical features on superhydrophobic surface upon UVO irradiation (Fig. 2B). The spectrum of the superhydrophobic substrate was divided into two peaks corresponding to C–H and C–O groups that are centred at 285.01 and 286.30 eV, respectively. The first component clearly dominates the C1s peak, being consistent with the

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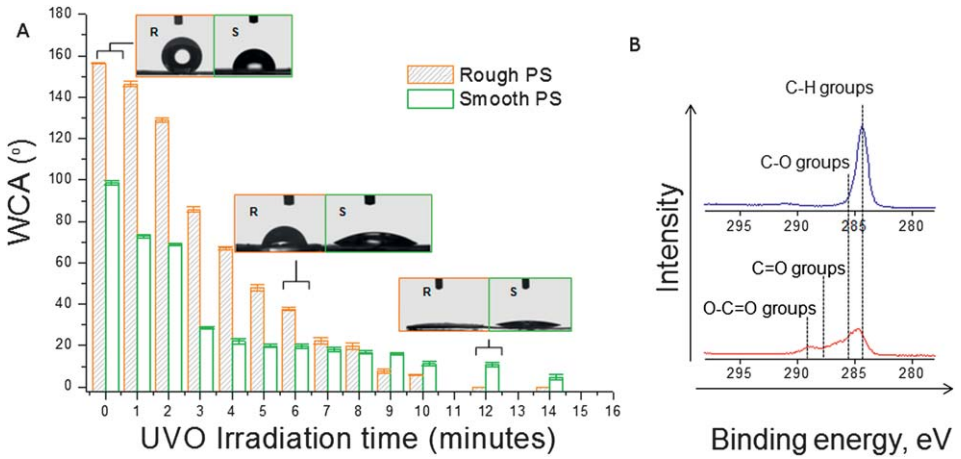

Fig. 1 (A) Wettability evolution measured by the water contact angle (WCA), as a function of UVO irradiation time for smooth and rough PS surfaces. The images show water droplets over the rough (right) and smooth (left) surfaces at three different levels of UVO irradiation. (B) XPS C1s spectra of the superhydrophobic surface (top spectrum) and of the same substrate after 12 minutes of UVO irradiation (bottom spectrum).

molecular structure of PS. Upon 12 minutes of UVO irradiation, two other contributions could be detected in the C1s peak, at 287.90 and 289.26 eV that attributed to C═O and O–C═O groups, respectively. The peak area corresponding to the C–H groups was substantially reduced (superhydrophobic: 96.1%; superhydrophilic: 57.6%) with the effect of the UVO irradiation. The results are consistent with the photochemical modification of the $–CH_3$ groups on the superhydrophobic surface into CHO, COOH and OH groups. The topography of the rough surface as observed by scanning electron microscopy (SEM) did not change substantially with UVO irradiation; therefore the changes observed in the WCA should be the result of the increase of hydrophilicity of the material upon UVO irradiation due to the introduction of oxygen-containing groups. The change of wettability in the rough PS surfaces should affect protein adsorption. Adsorption studies of both HSA and HFN were performed where adsorption kinetic data were obtained using solutions with different concentrations of the proteins put in contact with surfaces with distinct wettabilities (see Fig. S2 in the ESI†).

Using masks with *ca.* 1 × 1 mm^2 open square regions it was possible to pattern wettable hydrophilic spots onto the PS superhydrophobic substrates (Fig. 2A). The shape and spreading extend of a liquid confined inside such spots depend on both volume of the liquid and wettability on the spots (Fig. 2B). It is clear that even for the largest volumes, the liquid elements are well confined in the wettable spots, due to the strong difference in surface tension with the superhydrophobic surroundings. As expected, the droplets with the same volume are more spread in the spots subjected to longer UVO irradiation times (lower WCA).

4 μl of protein solutions with different concentrations were individually dispensed in the 1 × 1 mm^2 squares produced by patterning the superhydrophobic surface using 6 minutes of UVO irradiation. Different contact times of the droplets with the surface were also

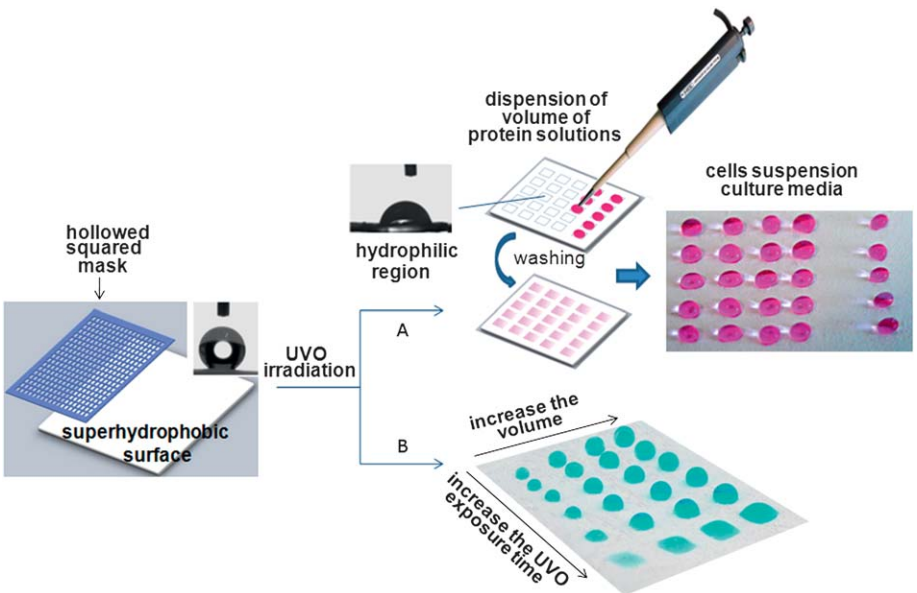

Fig. 2 (A) Schematic representation of the sequential steps used to produce the substrates and to employ them to test cell–protein interactions. (B) Droplets of different volumes from 2 to 8 μl confined in wettable regions produced by different UVO irradiation times from 1 to 12 min.

varied in one direction of the array. The fluorescent images of the surfaces after washing (Fig. 3Ai and ii) show the regions where HSA and HFN (green and red, respectively) were deposited. The amount of protein adsorbed is dependent on the protein concentration of the solution: in both proteins fluorescence intensity increases with increasing protein concentration. However, the increase in adsorption time has less influence on the fluorescence intensity of the protein adsorbed, especially after 30 minutes of contact. Both concentration and adsorption time effects observed by fluorescence in the spots of the array are consistent with the quantitative results obtained separately (see Fig. S2C and D in the ESI†)

Upon implantation different proteins from serum will adsorb onto the surface of biomaterials in a competitive way, the final composition of the protein layer formed will be determinant in the cellular events and the overall host response.[1] HSA is presented in high quantities in human blood plasma and HFN is known to facilitate cell attachment onto biomaterial surfaces. Thus, HSA and HFN are representative of a large number of serum proteins and it is reasonable to understand how cells react when they are previously incubated in solutions containing both kinds of proteins.[8]

The deposition of solutions containing different relative amounts and total concentrations of the HSA/HFN binary system was carried out in the developed arrays. After 2 hours of contact with a patterned array and washing these solutions containing fluorescent-labelled proteins the surfaces were observed by fluorescent microscopy (Fig. 3Aiii). As expected the fluorescent intensity increased with the total protein concentration and the coloured fingerprints are consistent with the relative composition of the two proteins.

Cell attachment to biomaterials will depend strongly on the surface characteristics, including the nature and organization of pre-adsorbed proteins. To demonstrate the applicability of the developed chips for HTS, the substrates prepared with the same combinations and quantities of HSA and HFN were used for the cell studies. 10 μl of culture medium containing 1000 cells were individually dispensed in each spot containing different combinations of pre-adsorbed proteins. After 4 hours of culture the chip was washed and cells were fixed and stained. The array was imaged using confocal microscopy (Fig. 3Bi). In a control column spots without any pre-adsorbed proteins were seeded with different number of cells. Image software was used to count the number of cells in each spot allowing to

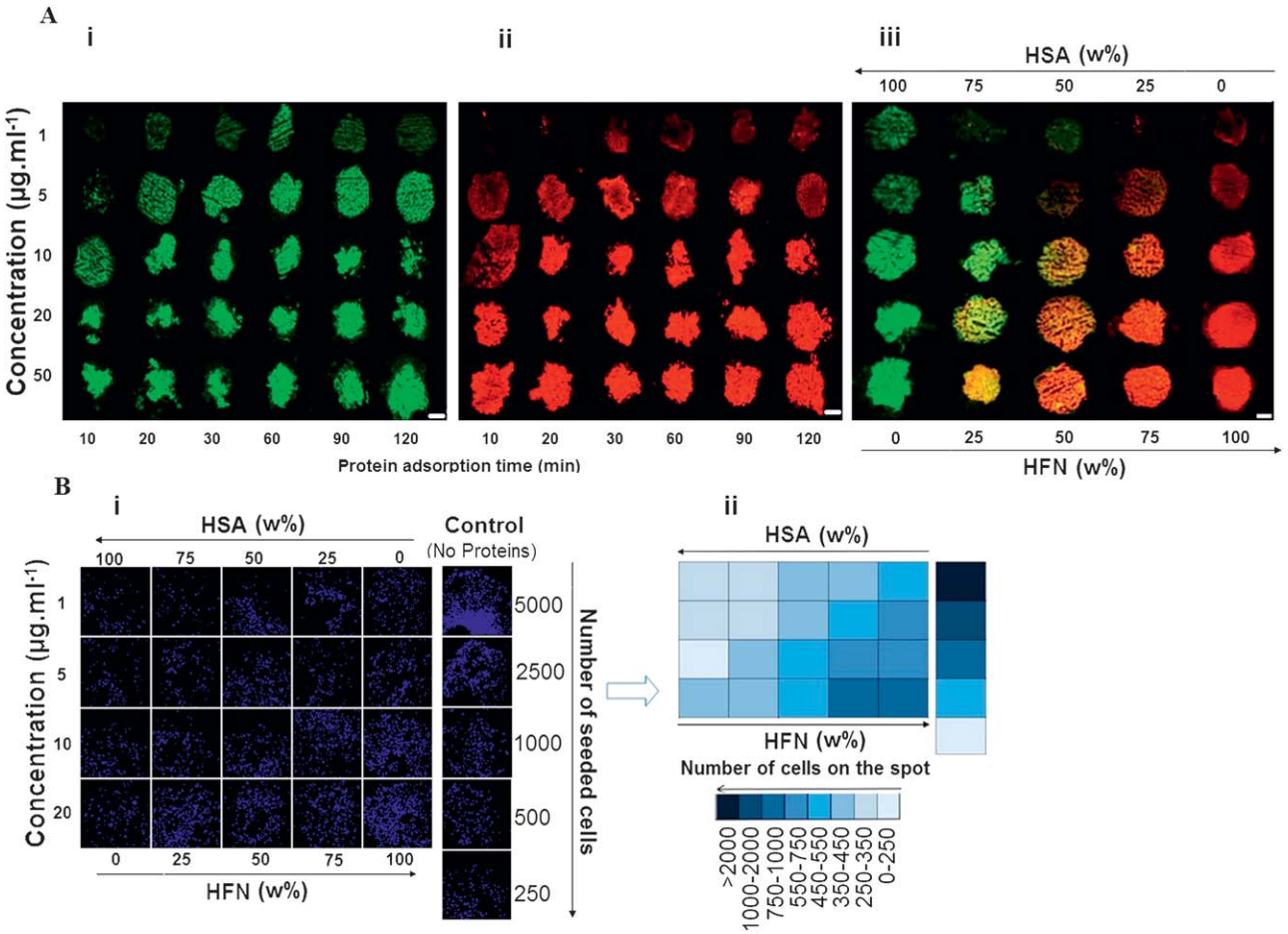

Fig. 3 (A) Fluorescence microscope images of substrates where spots were incubated with 4 μl solutions for 2 hours of (Ai) HSA (green) and (Aii) HFN (red) with different concentrations (vertical axis) and during different adsorption times (horizontal axis). (Aiii) HSA and HFN fluorescent fingerprints in patterned surfaces after different relative amounts and protein concentrations were deposited in the hydrophilic spots. (Bi) Confocal microscope pictures of osteoblast-like cells cultured for 4 hours on the micropatterned array pre-adsorbed with different protein quantities (equivalent to the array of Aiii). The column on the right represents the number of seeded cells attached on hydrophilic spots without the protein pre-adsorbed. (Bii) The heat map for the cell number per spot corresponding to the same array tested with different combinations of proteins. Scale bars, 500 μm.

generate an intensity map of cell number for the array tested (Fig. 3Bii). As a tendency, for the same total protein concentration, more cells are detected in the spots treated with higher relative HFN amounts. For the same HFN/HSA composition, the number of cells also tends to increase with increasing total protein concentration, also corresponding to an increase in the total amount of HFN. Such findings are consistent with the fact that albumin is a passivating protein and HFN has cell adhesive properties (including for SaOs-2[9]) due to the existence of integrin binding domains in its structure. In particular HFN presents RGD and PHSRN sequences with synergistic action able to modulate the biological activity of cells.[10] From the results of Fig. 3B one can conclude that the adsorbed HFN exhibited an adequate conformation to promote cell attachment. Other more complex combinations of materials could have been deposited in a larger array of spots as the patterning of hydrophilic regions could be easily extended to larger areas of the initial slide and robotic liquid handling could have been employed. The advantage of the method proposed is that different media or different numbers/types of cells could be deposited in each spot. Different polymeric biomaterials could be tested by dispensing distinct volumes of their solution in the array of spots. The materials could be then attached to the surface of each spot by adsorption, evaporation of the liquid followed by cross-linking or other methods to induce insolubility of the material.

In conclusion, we developed a concept based on the use of superhydrophobic flat substrates with controlled wettable spots to produce microarray chips to be used as a new low cost platform for high-throughput analysis that permits to screen the biological performance of combinations of biomaterials, cells and culture media. Such an inexpensive and simple bench-top method, or simple adaptations from it, could be integrated in tests involving larger libraries of substances that could be tested under distinct biological conditions, constituting a new tool accessible to virtually anyone to be used in the field of tissue engineering/regenerative medicine, cellular biology, diagnosis, drug discovery and drug delivery monitoring.

Experimental

Preparation of superhydrophobic substrate

Superhydrophobic polystyrene (PS) surfaces were processed using a one-step phase-separation methodology under ambient atmosphere. In our group, we have proposed a simple and economical phase separation method for producing such surfaces as supports for cell attachment studies, particles production or in microfluidics.[7,11] In a previous work we showed that hydrophilic or superhydrophilic regions could be produced on superhydrophobic polystyrene substrates through UV/Ozone (UVO) irradiation.[7] 70 mg ml^{-1} solution of polystyrene (PS commercial granules of an injection moulding grade) was prepared dissolving the granules in tetrahydrofuran (THF, from Sigma-Aldrich of Riedel-de-Haen) solvent for 2 hours to form a pre-solution at ambient temperature. Then, 1.3 ml of ethanol (from Panreac) was added into 2 ml of PS pre-solution and the mixed solution was stirred. A few drops of the mixture were dipped onto a cleaned PS film (purchased from Goodfellow, UK, with 0.25 mm of thickness, ref. ST311250/3). The substrate with the mixture was immersed in ethanol for 1 min. Afterwards the surface was dried under a nitrogen flow and a porous PS surface was obtained. The wettability was increased using a UVO high intensity mercury vapour lamp (254 nm) of a UV/Ozone ProCleaner device (Bioforce nanoscience, USA). The wettability of the surfaces studied was assessed by contact angle measurements. Static water contact angle (WCA) measurements were carried out using an $OCA15^{+}$ goniometer (DataPhysics, Germany) using the sessile drop method. Distilled water (6 μl) was dropped on the surfaces and pictures were taken after stabilization of the water drop. The surface morphology of the samples was analyzed using a Leica Cambridge S-360 scanning electron microscope, SEM (Leica Cambridge, UK). The surface chemistry of modified samples was investigated by X-ray photoelectron spectroscopy (XPS). XPS was performed using a VG Escalab 250 iXL ESCA instrument (VG Scientific) with monochromatic Al Kα radiation ($h\nu = 1486.92$ eV) and a takeoff angle of 90° relative to the sample surface. The measurement was carried out in constant analyzer energy (CAE) mode with a 100 eV pass energy for survey spectra and a 20 eV pass energy for high-resolution spectra. The C1s peak was resolved into three peaks at 285.0 eV.

Protein adsorption studies

The quantitative analysis of protein adsorption in the developed surfaces covering an extreme wettability range was made at five different concentrations: 10 μg ml^{-1}, 20 μg ml^{-1}, 30 μg ml^{-1}, 40 μg $ml^{-1}$and 50 μg ml^{-1}. The molecules selected for this study were human serum albumin (HSA, ≥95% pure, 30% aqueous solution, Calbiochem, US and Canada) and human plasma fibronectin (HFN, 95% pure, 0.1% Sigma-Aldrich, Germany). Circular samples (13 mm of diameter) previously subjected to UVO irradiation with different exposure times were placed in 24-well plates and 1 ml of HSA and HFN with different concentrations was deposited on each plate well. The analysis was also made at different protein adsorption times ranging from 10 min to 120 min. After 2h of incubation at 37 °C, the remaining protein (HSA and HFN) in solution was assessed by a colorimetric method for total protein quantification, using the Bradford assay (Sigma, Germany) and reading emission at 562 nm in a microplate reader (BioTek, USA). The protein concentration was determined by measuring the absorbance at 562 nm using a calibration curve for each protein. The amount of protein absorbed was calculated subtracting the obtained value of microplate reader with the initial value of protein put in contact with the substrate.

Biological tests on the developed substrates

Superhydrophobic-based microchips were prepared using a squared hollowed mask with open regions with 1×1 mm^2 size. In this particular work the microarrays are produced using PS that is applied commercially to produce tissue culture ware. To produce the patterns, the superhydrophobic substrates were covered with a mask with hollowed areas and exposed to 6 min of UVO irradiation. Controlling time exposure permits to control the wettability of the irradiated regions. Drops (4 μl) with different relative amounts and concentrations of fluorescent HSA and HFN were placed on the modified areas. HFN was purified from human plasma following the protocol of the Molecular Probes' Alexa Fluor 555 Protein Labeling Kit. For fluorescent detection, HFN was labeled by a fluorescent dye tetramethylrhodamine (TRITC) and HSA with isothiocyanate (FITC). Unlabelled albumin, fluorescein isothiocyanate conjugate bovine was purchased from Sigma and was diluted in

phosphate-buffered saline (PBS) shortly before use and adjusted to concentrations of 1 μg ml^{-1}, 5 μg ml^{-1}, 10 μg ml^{-1}, 20 μg ml^{-1}and 50 μg ml^{-1}. The fluorescence signal from the contact between the protein drop and the surface was recorded by a reflected/transmitted light microscope (Zeiss). The substrates were then washed gently with PBS. The intensity of the fluorescence signal from the proteins deposited on the superhydrophobic surface was used as a reference in order to compare the amount of protein molecules absorbed favourable for cell attachment. In a second part of this work we analyzed the effect of protein competition in the surface and evaluated the influence in cell adhesion and proliferation. Cell culture studies were performed with SaOs-2 human osteoblast-like cells (European Collection of Cell Cultures—ECCC, UK). Cells were expanded in DMEM (Sigma), supplemented by 10% foetal bovine serum (FBS; Invitrogen) and 1% antibiotic/antimycotic (Gibco). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 °C and culture medium was changed twice a week. Cultures were maintained until near confluence and then were enzymatically released (0.04% trypsin), counted using a Neubauer-counting chamber, seeded at a density of 1×10^5 cells per ml. Cell adhesion studies were performed by modifying the hydrophilic regions on the microchip with specific proteins to guide subsequent cell attachments. 10 μl of this cell suspension were dropped in each spot of the prepared microchip. Subcultures were characterized at 4 hours for cell adhesion/proliferation using fluorescence microscopy. For fluorescence staining, cells were fixed with 3.7% formaldehyde and rinsed with PBS. DAPI staining was used to label the DNA in the adherent cells. Briefly, the DAPI labelling solution 0.5 μg ml^{-1} was incubated for 1 min at room temperature in the dark. The microchips were washed in PBS to remove the remaining staining solution and imaged using a fluorescent confocal microscope (Olympus FluoView FV 1000). This microscope reveals the rearrangement of a number of such cells in each microchip spot with different combinations of HFN and HSA concentrations. Image analysis was used to count the number of cells in each spot. The same threshold criterions were used in all images.

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